terms, probably a mixture of lumi- and meta-rhodopsin (Wald, Durell & St George, 1950)—does not show indicator behaviour. If, in all these compounds, the terminal carbon atom of retinene is linked to a nitrogen atom of opsin by a double bond, then to account for their lack of indicator properties, the nitrogen atom must be either fixed in the quaternary state or not involved directly in the effective chromophore of rhodopsin.

SUMMARY

1. The stability of retinylidenemethylamine, an indicator yellow analogue, has been studied in aqueous solutions over a range of pH values.

2. It is concluded that retinylidenemethylammonium ions are stable, but that uncharged retinylidenemethylamine molecules hydrolyse to retinene. This hydrolysis is prevented by the presence of excess methylamine, but not by methylammonium ions.

3. These findings explain the influence of pH on the stability of indicator yellow solutions obtained from eyes.

4. On adding acid to rhodopsin solutions in the dark, stable acid indicator yellow solutions can be formed, with the retinene residue attached to the same nitrogen atom as it is in rhodopsin.

5. Essentially all the retinene residues in the rhodopsin chromophore have been shown to be attached to a nitrogen atom of opsin.

6. The status of the different types of indicator yellow formed from rhodopsin solutions is assessed.

We are indebted to the Medical Research Council for financial assistance.

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A Modified Conway Unit for Microdiffusion Analysis

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(*Received* 21 *June* 1954)

Absorption by the fixative is a difficulty in the microdiffusion method (Conway, 1950) for the determination of small amounts of bromide. Conway recommends a mixture of solid and liquid paraffin as fixative, but points out that the quality of the paraffin is important and that the glass lids should be only lightly smeared. However, it has been found that a definite amount of bromine (sometimes as much as 0·10 m-mole) was always absorbed by the paraffins available here. A similar difficulty with chloride determinations induced Gordon (1952) to use a chlorinated fixative. Pirt & Chain (1952) avoided such small losses of halogen by using a silicone fixative.

A slight modification of the unit is described which avoids any such difficulty and is also more suitable for analytical investigations where relatively high temperatures are required.

EXPERIMENTAL AND RESULTS

The modified unit has an extra chamber (the closing chamber) which is half-filled with the same solutions as the outer diffusion chamber, with the exception of the fluid to be analysed. (In the case of bromide determinations a saturated potassium dichromate solution in 20% (v/v) H_2SO_4 was used.) A liquid trap is formed by dipping an inverted Petri dish into this chamber and thus closing the Unit immediately after running-in the liberating substance. A similar principle of a liquid trap for closing the Unit was developed by Kinsey & Robison (1946), who used oil in the trap. Their Unit was, however, quite different from Conway's and consisted of only one diffusion chamber.

Figs. 1 and 2 show the modified Unit with the cover. In the Unit illustrated in Fig. 2 the closing chamber was made from acrylic plastic (Plexiglass) and attached to the Units by a plastic fixative. If Conway Units are not available for modification, Units containing all three chambers can easily be constructed of one material and no surface requires

grinding. Glass and porcelain have proved to be suitable, while many plastic materials are not always sufficiently inert. Porcelain units of the modified form (Fig. 1) have been supplied by Rudolph Grave, Stockholm.

To open the Unit it is most convenient to suck out the contents of the closing chamber before detaching the Petri dish. As it is sometimes difficult to grip the dish with the fingers, it was grasped with a rubber suction cup and lifted vertically. A piece of filter paper was then interspaced between the dish and the Unit so that it could be put aside without the risk of dropping any liquid into the inner chamber. The analyses then followed the descriptions by Conway (1950).

This modification excludes the possibility of gas leakage, and, as the liquid in the trap is also that used to liberate the volatile substance in the outer diffusion chamber the risk of absorption is eliminated.

Another advantage with this type of Unit is the ease with which it can be cleaned compared with those in which paraffin, vaseline or silicones were used. Washing with soap and then acid could be omitted. Using it for bromide determinations a short soaking in dichromate–sulphuric acid (20%, v/v, H_2SO_4) followed by rinsing in water was sufficient. Generally it is advisable to soak the unit in the same solution as is used for the liquid trap.

The theoretical treatment given by Conway (1950) is not significantly changed by introducing the closing chamber. Only the mean distance of passage in the gaseous phase between the outer and inner chamber will be a little increased, thus making the gradient of the gas tension somewhat lower. As, however, the time for passage in the air space is only a small fraction of the time necessary for the whole diffusion procedure it can be neglected in practice, and the final distribution of the analysed substance will not be significantly changed.

Experience with this new Unit has given excellent results with roughly the same clearance time as with the original Units.

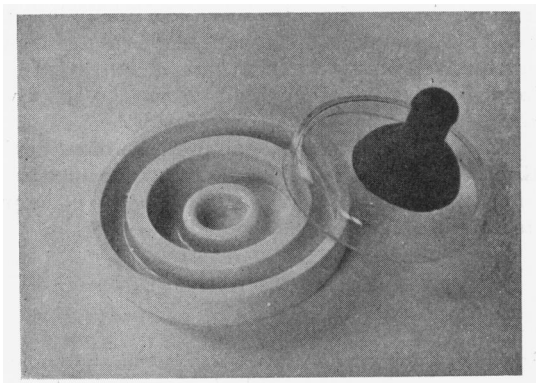

Fig. 1. Modified Unit with Petri dish and rubber suction cup.

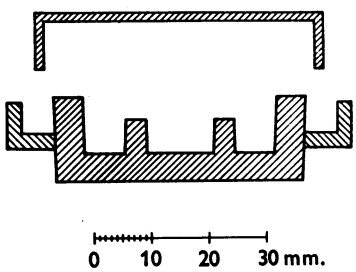

Fig. 2. Cross-section of the Unit and the dish.

In Figs. 3 and 4 calibration curves for bromide and ammonia determinations are shown. The bromide ion was oxidized by saturated potassium dichromate to bromine, which diffused into a solution of potassium iodide in the central chamber. The liberated iodine was then titrated by thiosulphate. For unknown reasons the calibration curve does not pass through the origin.

The ammonia was formed from an ammonium salt by saturated potassium carbonate and absorbed in a known amount of hydrochloric acid in the central chamber. The ordinate of Fig. 4 shows the difference in the amount of barium hydroxide required to neutralize the total amount of the

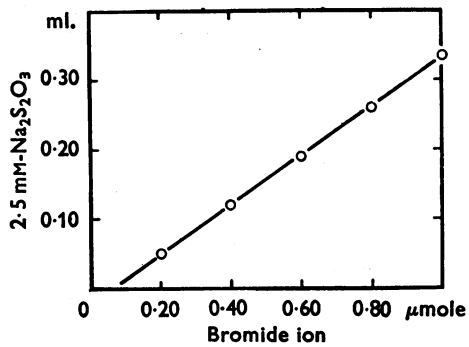

Fig. 3. Calibration curve for bromide determinations.

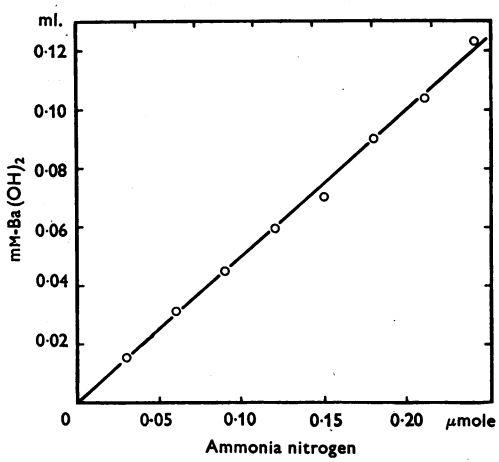

Fig. 4. Calibration curve for ammonia determinations. The ordinate gives the difference in ml. of base required for a blank and sample determination.

hydrochloric acid (blank) and the excess of the acid after absorption of the ammonia.

The proposed modification may be of use in many other analytical procedures involving the use of the Conway microdiffusion method.

SUMMARY

The ordinary Conway Unit has been modified by the addition of a peripheral chamber, to be filled with the same liquid as the outer diffusion chamber (except for the fluid to be analysed). An inverted Petri dish dipped into this chamber forms a liquid trap.

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The Isolation of 16-*epi*Oestriol from the Urine of Pregnant Women

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(*Received* 17 *August* 1954)

Many of those who have employed fluorescence reactions with sulphuric or phosphoric acids for the quantitative determination of the oestrogens have noted that phenolic fractions obtained from acid-hydrolysed human urine contain fluorogens other than oestriol (oestra-1:3:5-triene-3:16α:17β-triol), oestrone or oestradiol-17β (cf. Engel, 1950; Zondek & Finkelstein, 1952). While hitherto there has been no evidence to indicate that this 'non-specific' fluorescence is due to substances chemically related to the oestrogens, recent work suggests that it is in part due to substances closely resembling the oestrogens in their solubilities. Thus Migeon (1953), using countercurrent distribution methods, detected three unknown fluorogens in extracts from the urines of normal men and women and patients with hyperactivity of the adrenal cortex, and he showed that these had partition ratios intermediate between those of oestriol and oestradiol-17β in one of the solvent systems employed. More recently Braunsberg, Stern & Swyer (1954) have reported that all samples of crystalline oestriol examined by them contained a fluorogenic impurity which was eluted slightly in front of oestriol itself from partition chromatographic columns in which an alkaline aqueous stationary phase was employed.

Convincing evidence indicating the presence of a substance in human urine, chemically related to, but not identical with oestriol, oestrone or oestradiol-17β, has been obtained using the highly specific Kober reaction (as modified by Brown (1952) and by Bauld (1954)). Our colleague Dr J. B. Brown (personal communication) has detected the presence of a fourth Kober-chromogen in acid-hydrolysed human pregnancy urine by experiments involving chromatography on alumina columns of methylated phenolic fractions. This fourth Kober-chromogen was found by him to be eluted less readily than the 3-methyl ether of oestradiol-17β, but more readily than the 3-methyl ether of oestriol. Working independently, one of us (W.S.B.) detected a fourth Kober-chromogen in the oestriol fraction obtained from the urine of a non-pregnant woman. This unknown chromogen was eluted from partition chromatographic columns somewhat in front of oestriol using the system 70% (v/v) aqueous methanol–ethylene dichloride.

The evidence indicating the presence of a fourth Kober-chromogen (KC-4) in human urine appeared to be so convincing that it was decided to attempt its isolation and identification.

RESULTS

Isolation of KC-4

In the first instance several small batches of pooled pregnancy urine were worked up in order to develop methods for the concentration of KC-4 which could be conveniently used in subsequent large-scale experiments. For the detection of KC-4 in these trial fractionations column partition chromatograms, using the system 70% methanol on Celite–ethylene dichloride (Bauld, 1953), were employed.

Oestriol fractions containing KC-4 were prepared from ether extracts of the acid-hydrolysed urine both by the usual benzene–water partition procedure and by the original procedure of Cohen & Marrian (1934) involving extraction of a 'strong phenolic' fraction by 0·1 N-NaOH from ether. The latter procedure, being more economical in solvents, was clearly preferable for large-scale use.